Biochimica et Biophysica Acta, 766 (1984) 501–504
Elsevier

BBA Report

BBA 40040

HIGH RESOLUTION OPTICAL SPECTRA IN VIVO

PHOTOACTIVE PROTOCHLOROPHYLLIDE IN ETIOLATED LEAVES AT 5 K

INDREK RENGE, KOIT MAURING and REIN AVARMAA

Institute of Physics, Estonian Academy of Sciences, Riia 142, 202400 Tartu, Estonian SSR (U.S.S.R.)

(Received March 5th, 1984)

Key words: Protochlorophyllide; Fluorescence spectroscopy; Site selection spectroscopy; Chlorophyll; (Etiolated barley leaf)

We describe here high-resolution spectra of etiolated leaves studied by means of selective monochromatic excitation of fluorescence as well as by hole-burning technique at liquid helium temperature. Up to now site selection spectroscopy has not been successfully applied to biologically active chromophores in vivo. Our results demonstrate that even in the presence of specific pigment-protein and pigment-pigment interactions very narrow purely electronic lines can be obtained in the optical spectra of biological systems.

It has been shown [1] that sharp-line fluorescence spectra with a resolved vibrational structure can be obtained using selective laser excitation of organic molecules in solid solutions as a result of the elimination of large inhomogeneous broadening. This technique ('site-selection spectroscopy') was successfully used for revealing fine-line vibronic fluorescence and excitation spectra at liquid helium temperature, and detailed information about their vibrational structure was delivered for chlorophyll *a* and its closest analogs [2–6]. A photochemical hole-burning method [7,8] enabled to improve further the resolution (up to $5 \cdot 10^{-3}$ cm^{-1}) in the chlorophyll spectra [6,9].

However, the attempts to obtain fine-line spectra for in vivo systems by selective excitation remained unsuccessful. In has been proposed [3] that the main reasons of the absence of site selection effect for chlorophyll in green leaves are strong pigment-protein interactions and energy transfer, leading to spectral diffusion. In recent studies of some pigment-protein complexes, sharp-line vibronic fluorescence spectra were obtained for solubilized iron-free cytochrome *c* [10], while hole-burning was observed in the absorption bands of dissolved covalently bound biliproteins [11] as well as for *Chlamydomonas* cells [12].

Recently, we observed [13] sharp-line fluorescence excitation spectra for photochemically inactive (quasi-free) protochlorophyllide ($PChl_{630/635}$, the numbers denote in vivo absorption and fluorescence maxima, respectively) and chlorophyll(ide) in etiolated and greening leaves, i.e., at a low level of pigment concentration. At the same time the spectra of photoreducible $PChl_{650/656}$ remained practically structureless. Thus, the question about the possibility of obtaining high resolution spectra for biologically active chromoproteids remained open.

Here we report the results demonstrating that the formation of photoactive aggregated pigment-protein complexes is not the principal obstacle in observing highly resolved spectra in vivo.

In the first series of measurements a spectrum hole-burning was performed in the main absorption band of $PChl_{650/656}$ of etiolated barley leaves (Fig. 1). A narrow hole at the frequency of the dye

Abbreviation: PChl, protochlorophyllide.

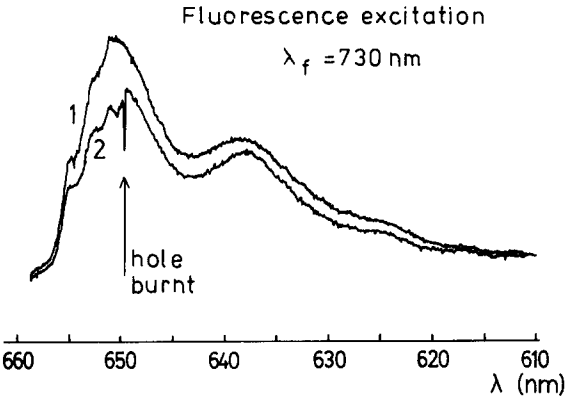

Fig. 1. Fluorescence excitation spectra of a 12-day old etiolated barley leaf at 5 K scanned with a Coherent CR-490 dye laser at 0.2 $mW \cdot cm^{-2}$ constant light density and 0.5 cm^{-1} linewidth: before (1) and after (2) hole-burning at 649.5 nm by 2 min exposure to 200 $mW \cdot cm^{-2}$ laser power. Fluorescence was detected with a 20 cm^{-1} bandpass in the vibronic transition region of protochlorophyllide pigments at 730 nm.

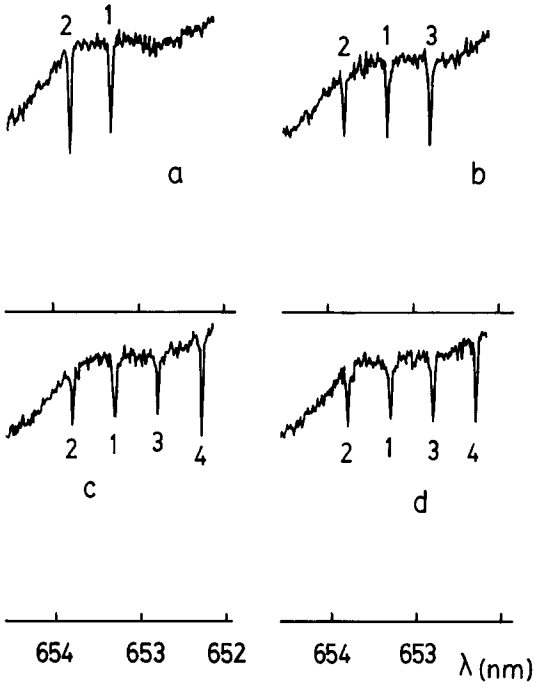

Fig. 2. Multiple hole-burning in the fluorescence excitation spectra of a 14-day old etiolated barley leaf (conditions as in Fig. 1). The holes were consecutively burnt in the order from 1–4 and spectra taken in the order from a–d. Between the spectra c and d, there was a 10 min dark period, while the scanning of one spectrum lasted for 2 min.

laser corresponds to a purely electronic S_1-S_0 transition in the $PChl_{650/656}$ chromophore. Fluorescence excitation was used as a sensitive probe of absorption, but it was checked that a hole of about the same depth appeared in the absorption band as well. The hole-burning process is due to the selective absorption of the laser radiation via resonant no-phonon lines followed by some photochemical transformation. Most remarkable is the formation of a rather deep (up to 40% of the initial intensity) purely electronic hole with a weak phonon wing (seen in Fig. 1 as a shallow side-hole). The high line-to-wing intensity ratio indicates a weak electron-phonon coupling which may result from a relatively rigid environment of the chromophores. Still, interaction with low-frequency vibrations whose energy is comparable with kT can be responsible for the estimated width $\delta\nu \approx 0.3$ cm^{-1} of the holes we observed at 5 K.

Some measurements were carried out to clarify the mechanism responsible for the hole-burning process. At 5 K the hole was found to be stable, except for some filling-up by 20–25% during a 10 min dark period (Fig. 2c and d). Multiple hole-burning could be performed without any remarkable filling-up previously burnt holes, apart from the dark recovery (Fig. 2a, b and c). Thus, the mechanism seems to be different from the site redistribution (non-photochemical) one found in case of chlorophylls in frozen solutions [6]. Since a diminishing of the whole $PChl_{650/656}$ band took place after a prolonged exposure to the bruning light, there must exist a photochemical pathway leading to a species with different spectra properties. It is known that a non-fluorescent photoconversion product of $PChl_{650/656}$ called P-690 can be formed at low temperatures [14]. Although its formation was observed even at 77 K, the efficiency of the process was greatly diminished [15,16]. As we did not directly detect P-690 absorption, the observed photobleaching of $PChl_{650/656}$ at 5 K may be connected with the formation of earlier non-fluorescent intermediate(s) as well.

The poor vibronic structure in the excitation spectra of protochlorophyllide holochrome [13] could be explained as the result of energy transfer, for instance, between inactive $PChl_{630/635}$ and $PChl_{650/656}$. This effect would partially be

eliminated under resonant excitation within the 650 nm absorption band, when the ground state vibrational satellites in the fluorescence spectra are recorded. Indeed, when the exciting beam was sufficiently weakened and its wavelength slowly tuned in order to avoid burning, a reasonably good line structure was obtained (Fig. 3). A comparison with a solution spectrum in ether [5] reveals clear differences between in situ and in vitro PChl. Although the main vibrational groups (265–378, 740–795, 994–1000) cm^{-1}, etc.) are found in both spectra, the relative intensity of the lines differ significantly. The lines 762 and 795 cm^{-1} in solution have undergone particularly strong frequency shifts: -16 and -19 cm^{-1}, respectively. Rather strong specific pigment-protein interactions can account for these marked shifts in the vibrational frequencies of the fluorophore.

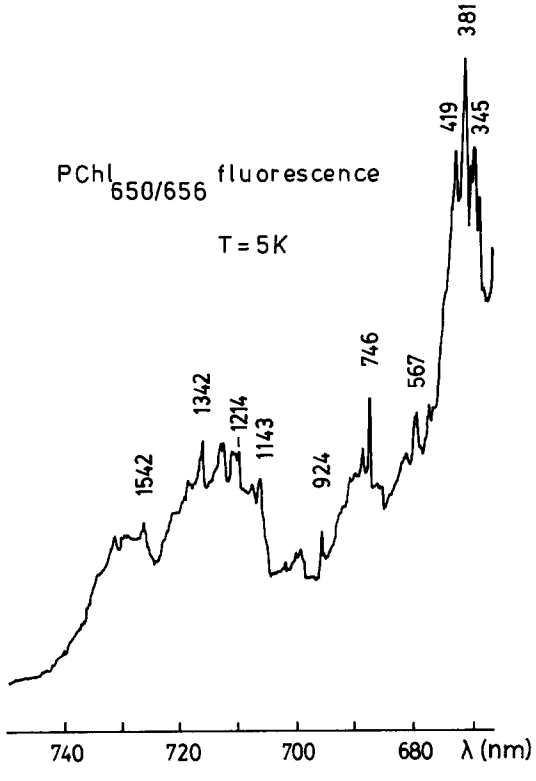

Fig. 3. Fluorescence spectrum of a 7-day old barley leaf excited under power 0.2 $mW \cdot cm^{-2}$. The excitation wavelength was slowly scanned in the interval 649–650 nm, which was taken into account at the calculation of the vibrational frequencies indicated near the lines.

To our opinion, results presented above demonstrate new possibilities in the sharp-line spectroscopy of in vivo systems frozen to liquid helium temperature. First of all, vibrationally resolved spectra should be obtainable for different fluorescent biologically active chromophores. Especially fruitful seems to be the hole-burning method as a very sensitive indicator of low-temperature photochemical transformations. The extreme narrowness of the purely electronic lines observable as spectral holes makes it possible to detect the influence of different external or internal fields, to follow subtle pigment-protein or pigment-pigment interactions, etc., as pointed out in a preliminary discussion below.

Exact measurements of the hole-width at lower temperatures could distinguish glass-like or crystal-like behaviour of the protein moiety of the PChl chromophore, since the temperature dependence of the linewidth has been found to be rather critical [17].

From the single line feature of the narrow 00-hole in the $PChl_{650/656}$ band, we get some information concerning the pigment-pigment interaction in protochlorophyllide holochrome. It has been shown that $PChl_{650/656}$ contains pigment molecules in an aggregated form, probably as dimers or tetramers [18], whereas the pigment-pigment interaction in aggregates is known to cause specific changes in their energy levels. As no explicit splitting of the purely electronic line was observed, we conclude that either the 'exciton splitting' in this complex is smaller than the observed linewidth of 0.3 cm^{-1}, or one of the components has a forbidden transition moment. This point certainly needs more careful consideration using higher resolution.

The authors thank K.K. Rebane for his continuous interest in our study.

References

1 Personov, R.I., Al'shits, E.I. and Bykovskaya, L.A. (1972) Optics Commun. 6, 169–173
2 Avarmaa, R. (1974) Eesti NSV Tead. Akad. Toimet. Füüs. Matem. 23, 93–94
3 Avarmaa, R. and Rebane, K. (1975) Stud. Biophys. (Berlin) 48, 209–218
4 Fünfschilling, J. and Williams, D.F. (1975) Photochem. Photobiol. 22, 151–152

5 Bykovskaya, L.A., Litvin, F.F., Personov, R.I. and Rmanovskii, Yu. V. (1980) Biofizika 25, 13–20
6 Rebane, K.K. and Avarmaa, R.A. (1981) J. Photochemistry 17, 311–317
7 Gorokhovski, A.A., Kaarli, R.K. and Rebane, L.A. (1974) Pis'ma JETP 20, 474–479
8 Kharlamov, B.M., Personov, R.I. and Bykovskaya, L.A. (1974) Optics Commun. 12, 191–193
9 Avarmaa, R., Mauring, K. and Suisalu, A. (1981) Chem. Phys. Lett. 77, 88–92
10 Angiolillo, P.J., Leigh, J.S. and Vanderkooi, J.M. (1982) Photochem. Photobiol. 36, 133–137
11 Friedrich, J., Scheer, H., Zickendraht-Wendelstadt, B. and Haarer, D. (1981) J. Am. Chem. Soc. 103, 1030–1035
12 Maslov, V.G., Chunaev, A.S. and Tugarinov, V.V. (1981) Molek. Biol. 15, 1016–1027
13 Avarmaa, R., Renge, I. and Mauring, K. (1984) FEBS Lett. 167, 186–190
14 Raskin, V.I. (1976) Vestsi Akad. Navuk BSSR 5, 43–46
15 Dujardin, E. and Correia, M. (1979) Photobiochem, Photobiophys. 1, 25–32
16 Litvin, F.F. and Ignatov, N.V. (1980) Dokl. Akad. Nauk SSSR 250, 1463–1465
17 Thijssen, H.P.H., Van den Berg, R. and Völker, S. (1983) Chem. Phys. Lett. 97, 295–302
18 Canaani, O.D. and Sauer, K. (1977) Plant Physiol. 60, 422–429